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Early effects of gamma rays and protons on human melanoma cell viability and morphology

D. TODOROVIĆ*, I. PETROVIĆ*, M. TODOROVIĆ†,
G. CUTTONE‡ & A. RISTIĆ-FIRA*

**Vinča Institute of Nuclear Sciences, Laboratory of Molecular Biology and Endocrinology, P.O. Box 522, 11001 Belgrade, Serbia*

†Faculty of Medicine, University of Kragujevac, Serbia

‡Istituto Nazionale di Fisica Nucleare, Laboratory Nazionali del Sud, Catania, Italy

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Summary

The effects of irradiation with gamma rays and protons on HTB140 human melanoma cell morphology and viability were analyzed. Exponentially growing cells were irradiated close to the Bragg peak maximum of the 62-MeV proton beam, as well as with ^{60}Co gamma rays, with doses ranging from 8 to 24 Gy. The overall cell morphology was unchanged 6 and 48 h after gamma irradiation, also showing a relatively weak cell-inactivation level. After exposure to proton beam, considerable changes in cell morphology followed by stronger cell inactivation were achieved. Proliferation capacity of irradiated cells significantly decreased in both experimental set-ups. Higher ionization level of protons with respect to gamma rays, representing the main physical difference between these two types of radiation, was also revealed on the cell membrane level through larger pro-apoptotic capacity of protons.

Introduction

Ionizing radiation is a DNA-damaging agent that is widely used in cancer therapy. This type of radiation produces DNA lesions, such as single and double strand breaks. Radiation also destroys the cell membrane by activation of sphingomyelinase, an enzyme that catalyzes the hydrolyses of sphingomyelin to the lipid second messenger ceramide, thereby inducing the cell death by apoptosis (Cohen-Jonathan *et al.*, 1999). The nucleus is another critical target for radiation-induced apoptosis, via activation of pro-apoptotic genes (Goodhead, 1999). Morphological changes resulting in apoptosis, such as shrinkage of cell volume accompanied by dilatation of endoplasmic reticulum and convolution of the plasma membrane, were described using light and electron microscopy. On the ultra-structural level of cell organization, early events in apoptotic process include loss of cell junctions and other specialized plasma membrane structures such as microvilli. Apoptotic cells break up into series of membrane-bound spherical bodies, containing structurally normal, but condensed organelles. The nucleus undergoes a profound, initially discontinuous chromatin condensation around the nuclear periphery (Cohen-Jonathan, 1999). Biochemical features of apoptosis include fragmentation of nuclear DNA, translocation of phosphatidylserine to the outer plasma membrane and activation of caspases that act as the signal transduction molecules (Norbury & Zhivotovsky, 2004). Contrary to the necrotic cells, which are lysed and destroyed with subsequent diffusion of inflammatory molecules, the apoptotic cells activate pathways leading to their suicide or self-elimination followed by macrophage phagocytosis.

In the past decades, therapeutic proton beams were successfully used in treating several tumour types. The advantage of proton beams compared with conventional therapy lies in their physical properties, such as well-defined range, relatively small lateral scattering and localized high ionization density, that is energy deposition (high LET [linear energy transfer]) just before the end of the range (Belli *et al.*, 2000). These characteristics make them suitable for therapeutic application in the cases where there is a high demand to minimize the destruction of the neighbouring healthy tissue and organs. The aim of this study was to investigate early cellular responses to conventional gamma irradiation and proton irradiation, considering changes in cellular morphology and viability of HTB140 human melanoma cells. The level of cell inactivation was additionally analyzed by following cell proliferation capacity and apoptotic death.

Correspondence to: D. Todorovic. Tel/fax: +381-11-2455-561; e-mail: dtodorovic@medf.kg.ac.yu

Material and methods

Cell line and irradiation conditions

Human HTB140 melanoma cells obtained from Human Tumor Cell Bank (HTB) of the American Type Culture Collection (ATCC, Manassas, VA) were used in this study. Cells were maintained in RPMI 1640 medium, supplemented with 10% foetal calf serum, penicillin–streptomycin and L-glutamine (Sigma Aldrich Chemie GmbH, Steinheim, Germany), under standard conditions (37°C, 5% CO_2). Exponentially growing cells were irradiated close to the Bragg peak maximum of 62 MeV protons at the CATANA (Centro di Adro Terapia e Applicazzioni Nucleari Avanzati) treatment facility, in the Istituto Nazionale di Fisica Nucleare, Laboratori Nazionali del Sud (INFN-LNS), in Catania, Italy. Delivered doses ranged from 8 to 24 Gy, at the dose rate of 15 $Gymin^{-1}$. Reference irradiations with conventional ^{60}Co gamma rays, at the same dose level and dose rate of 1 $Gymin^{-1}$, were performed at the Vinca Institute of Nuclear Sciences, Belgrade, Serbia. Cell number and viability were estimated 6 and 48 h after irradiation, using 0.4% trypan blue staining. Viable cells were counted in a haemocytometer, under light microscope at 100× magnification.

Light microscopy

The cellular morphology of monolayer cultures, 6 and 48 h after irradiation, was examined under inverted light microscope with phase contrast (Olympus Vanox, Tokyo, Japan) and CCD camera (JVC TK-C 1381, Digital $^1/_2$ Inch CCD) at 100× magnification, using Leica Qwin, Live computer program.

Cell proliferation assay

Enzyme-Linked ImmunoSorbent Assay (ELISA) is a biochemical technique used mainly to detect the presence of an antibody or an antigen in an analyzed sample. In our experiments Cell Proliferation ELISA, BrdU (colorimetric) Kit (Roche Diagnostics GmbH, Mannheim, Germany) was used for immunocytological detection of cells undergoing DNA replication. The proliferation capacity of irradiated cells was evaluated by measuring the level of incorporation of thymidene analogue 5-bromo-2-deoxyuridin2 (BrdU) into the DNA molecules during duplication. Assay was performed 6 and 48 h after irradiation, according to the manufacturer's instructions. The absorbance was evaluated using VICTOR2 1420 Multilabel counter (Wallac, Perkin Elmer, Turku, Finland), at test wavelength of 450 nm and reference wavelength of 690 nm. The developed colour and thereby the absorbance was directly correlated to DNA synthesis and hereby to the number of proliferating cells. Inhibitory rates were calculated according to the formula: inhibitory rate = (1 − tested cell proliferation/mean of control cell proliferation) × 100%.

Flow cytometric analysis

The quantification of apoptotic cells was assessed using a fluorescence activated cell sorter (FACS) (Becton, Dickinson, Heidelberg, Germany). In principle, the flow cytometry is a technique for counting, examining and sorting of particles (cells) suspended in a stream of fluid. The number of apoptotic cells was estimated by Annexin-V-FLUOS Kit (Roche). Annexin is a protein with high affinity and selectivity for phosphatidylserine, thus enabling the quantification of apoptotic cells through the detection of presence of phosphatidylserine on the outer side of the plasma membrane. According to the technical features of FACS, in each sample a population of about 10000 cells was gated. Apoptotic population was calculated using CellQuest computer program.

Statistical analysis

The independent Student's t test was performed to assess the significance of differences among different experimental groups. The level of significance was set at $P < 0.05$. Results are presented as the mean ± SD. Triplicate measurements were made during each experiment, and each experiment has been repeated three times, unless otherwise indicated in the text.

Results and discussion

In the present study, early effects of gamma rays and protons on melanoma cell morphology and growth were analyzed. Changes in morphology of irradiated HTB140 cells are shown in Fig. 1. It was observed that when grown under standard conditions, confluent untreated HTB140 cells display a polygonal shape. They grow in a predominantly fusiform morphology having round or oval nuclei with multiple small irregular nucleoli and pale-staining chromatin. After exposure to gamma irradiation, this cellular morphology generally remained unaffected even with the increase of the dose at both time points. By contrast, proton irradiation provoked changes in the cell morphology, monitored by membrane ruffling and loss of cell junctions (Fig. 1). With the increase of dose a more severe damage to the cell membrane was detected. This effect was particularly more pronounced 48 h after irradiation. Phase-contrast microscopy revealed changes in the cellular morphology characteristic of apoptotic cell death. Changes in cell shape, volume, membrane integrity and even in chromatin condensation were observed in such irradiated cells.

Gray structures noticed near the edge of cells, near the inner part of the membrane, could be attributed to the condensation of chromatin. Many round-shaped melanoma

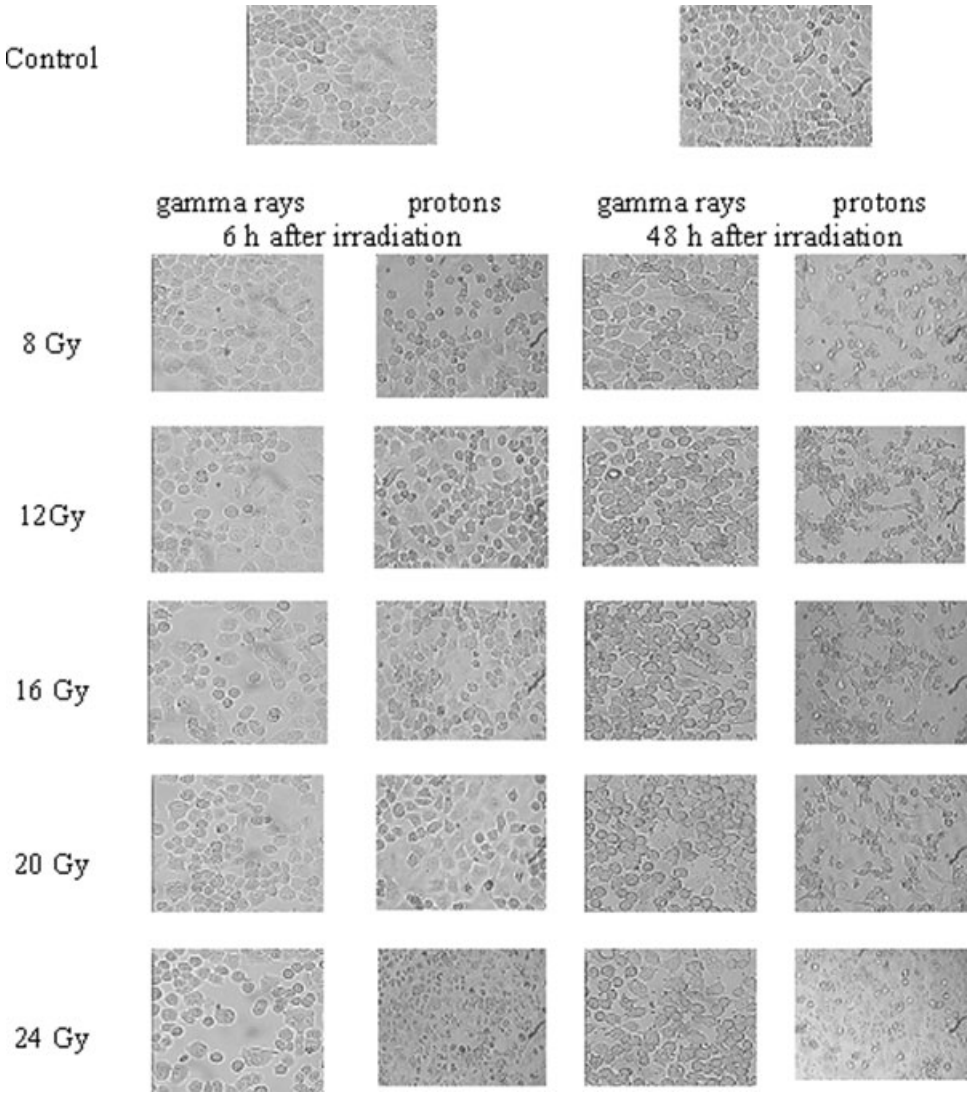

Fig. 1. Morphology of HTB140 melanoma cells 6 and 48 h after irradiation with gamma rays or protons in the dose range from 8 to 24 Gy. After gamma irradiation, cell morphology remained unchanged compared with non-irradiated controls having a polygonal shape. Protons produced morphological changes that included membrane ruffling and loss of cell junctions. Phase contrast magnification 100×.

cells, being probably pre-apoptotic, were detected 6 h after proton irradiation. Similar changes of cellular morphology were already reported for different cell lines such as Chinese hamster V79 and FRTL-5 cells, after being exposed to ionizing irradiations (Aoki *et al.*, 2000; Green *et al.*, 2002). All these morphological changes were followed by the significant reduction of the cell number.

Cell inactivation level was determined by the conventional trypan blue staining, based on the ability of viable cells, having unaffected cell membrane, to remain uncoloured in the presence of blue dye. Because of the destruction of cell membrane, dead cells are coloured blue. After gamma irradiation, a relatively weak inactivation of HTB140 cells was observed at both time points (Fig. 2). Owing to the specific distribution of dose, at the same dose level, a significantly stronger response was achieved after irradiation with protons. The level of cell inactivation of proton-irradiated cells compared with non-irradiated controls or gamma-irradiated cells was highly significant in the whole range of doses applied ($P < 0.01$; Fig. 2). Other colorimetric methods, predominantly based on metabolic activities of viable cells, such as MTT (3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide) assay as well as SRB (sulforhodamine B) assay, also supported these results (Ristic-Fira *et al.*, 2007).

Both protons and gamma rays significantly reduce proliferation capacity, 6 h after irradiation ($P < 0.05$; Fig. 3). At 48 h after irradiation, for the same dose range of each radiation type, a rather linear and highly significant decrease of cell proliferation was observed ($P < 0.001$).

Detected decrease of cell proliferation pointed out by the low level of incorporation of BrdU during cell replication

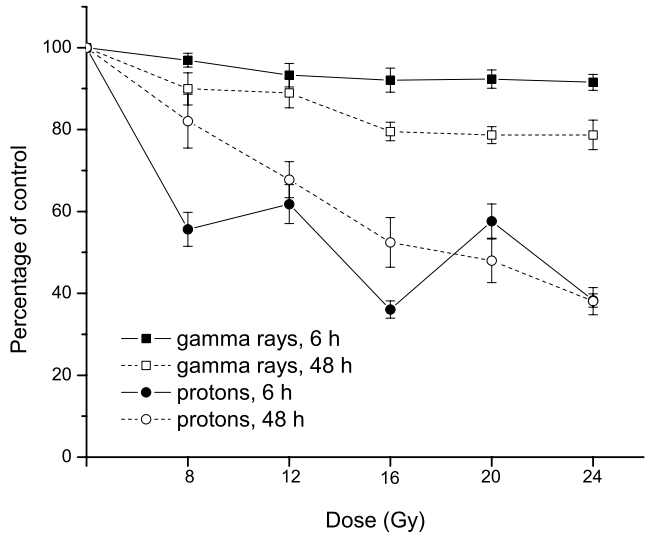

Fig. 2. Viability of HTB140 melanoma cells, 6 and 48 h after irradiation with gamma rays or protons evaluated under trypan blue exclusion. The level of cell inactivation was highly significant in the whole range of doses applied after proton compared with gamma irradiation ($P < 0.01$). Results from three separate experiments are presented as percentage of control (Mean ± SD).

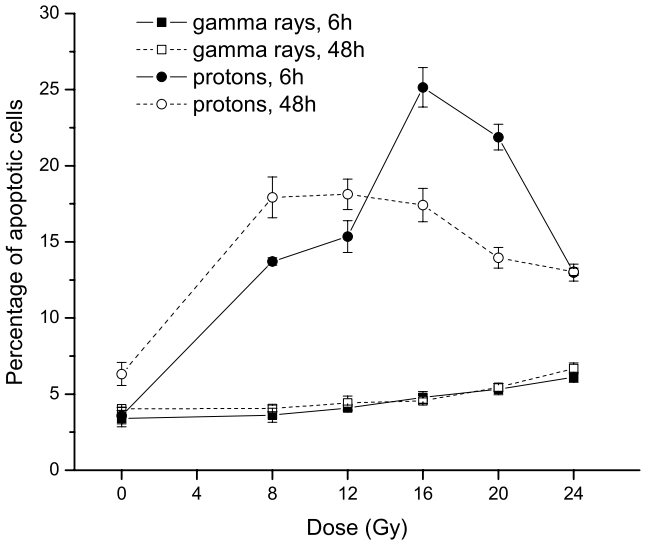

Fig. 4. Dose-dependent apoptotic cell death of HTB140 melanomas, 6 and 48 h after being irradiated with gamma rays or protons. Gamma irradiation induced less than 6% of apoptotic cells, in the whole range of doses applied, at both time points. Number of apoptotic cells, 6 h after proton irradiation was significantly higher with maximal value being 25% for 16 Gy protons. With prolonged post-irradiation incubation, up to 48 h, the number of apoptotic nuclei slightly decreased (Mean ± SD).

could be explained by the quantity and quality of damage produced by each irradiation. Triggered repair mechanisms as well as cell cycle arrest were initiated by the radiation and are proportional to their damaging ability (Karjalainen *et al.*, 1999).

Experimental results have also shown that protons produced changes on the cell membrane, such as translocation of phosphatidylserine (PS) from the inner part of the plasma membrane to the outer layer, leading to early apoptotic cell death (Fadok *et al.*, 1992). Phosphatidylserine (PS) positive cells, that is apoptotic cells, were detected by annexin-V-FLUOS assay and quantified by FACS (Fig. 4). The number of apoptotic cells, evaluated 6 h after proton irradiation, reached a maximal value of 25% for 16-Gy protons. With a prolonged post-irradiation incubation time to 48 h, the

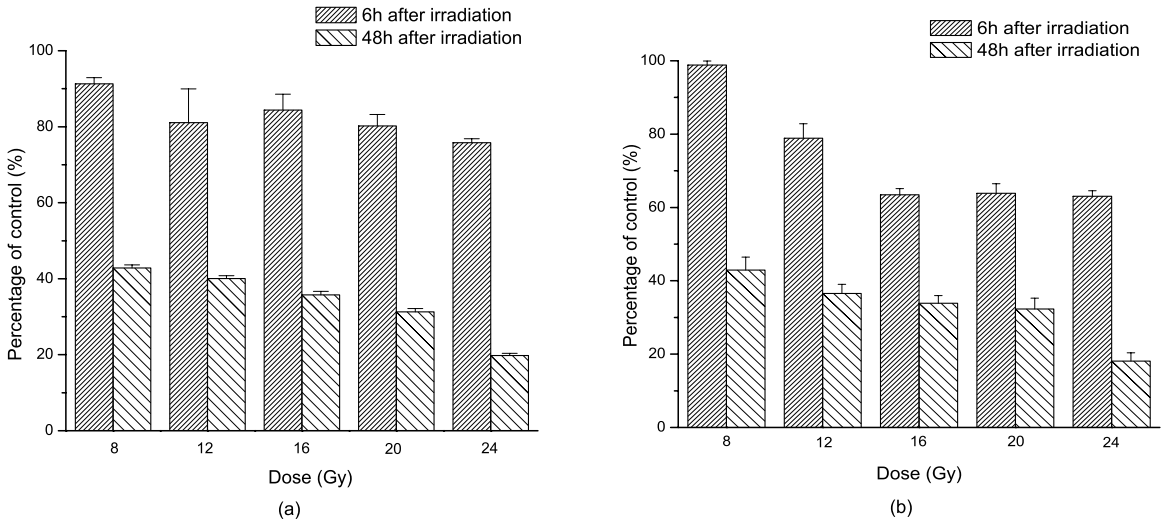

Fig. 3. Proliferation capacity of HTB140 cells, 6 and 48 h after irradiation with gamma rays (panel *a*) and protons (panel *b*), estimated by Cell Proliferation ELISA BrdU (colorimetric) Kit. Significant decrease of cell proliferation capacity was observed 6 h after irradiation with either gamma rays or protons ($P < 0.05$). At 48 h after irradiation, for each radiation type, almost linear and highly significant decrease of cell proliferation was detected ($P < 0.001$). Results from three separate experiments are presented as percentage of control (Mean ± SD).

number of apoptosis slightly decreased (Fig. 4). However, previously reported results indicated that gamma irradiation of HTB140 cells induced a low level of apoptosis, being less than 6% in the whole range of doses applied and at both time points (Petrovic *et al.*, 2006). The estimated level of irradiation-induced apoptosis of HTB 140 cells was in the range of reported values for other melanoma cell lines such as Me45 and HTB63 cells (Ristic-Fira *et al.*, 2001; Kumala *et al.*, 2003).

Although being at the limit of cellular radio-resistance, HTB140 cells exhibited a larger inactivation level and underwent morphological changes and apoptosis after being exposed to protons. Taking into account the therapeutic application of protons and their advantages over conventional radiation sources, such as ^{60}Co gamma rays, the reported results support their use even for the limited cases that are resistant HTB140 melanoma cells.

Conclusions

Magnitude and qualitative features of damages induced by gamma rays and protons were detected 6 and 48 h after irradiation using several biological end points. Significantly different responses, based on different radiation qualities, were detected, being more pronounced in proton-irradiated cells. Specific morphological changes followed by apoptotic cell death and stronger inactivation level occurred after proton irradiation. The level and type of observed morphological changes were in agreement with the number of apoptotic cells after proton irradiation.

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